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An economical and preparative orthogonal solid phase synthesis of fluorescein and rhodamine derivatized peptides: FRET substrates for the *Staphylococcus aureus* sortase SrtA transpeptidase reaction†

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An economical and preparative-scale orthogonal solid-phase method of incorporating carboxyrhodamine and carboxyfluorescein fluorescence resonance energy transfer (FRET) probes site-specifically into synthetic peptide substrates for the *S. aureus* Sortase transpeptidase SrtA has been developed.

Virulence in *Staphylococcus aureus* bacteria is conferred in part by the presentation of protein virulence factors that are covalently anchored to the bacterial cell surface.[1] These proteins are responsible for numerous virulence functions, including adhesion, colonization and evasion from the host's immune defenses.[1] The Sortase family of transpeptidases is responsible for the covalent attachment of virulence factors to peptidoglycan (Fig. 1).[2] In *S. aureus*, Sortase isoform A (SrtA) binds to substrates containing an LPXTG motif and cleaves the Thr-Gly bond with concomitant capture of the enzyme-acyl intermediate by nucleophilic attack of the α-amino group of Gly_5, the pentapeptide that cross links two peptidoglycan strands.[3–5] Sortases have emerged as targets for the development of a new class of chemotherapeutics that do not kill bacteria, but rather disrupt their ability to colonize and propagate within a host.[6–8]

Since numerous isoforms of Sortase exist among Gram-positive bacteria,[9] we recently developed a FRET-based activity assay for SrtA that monitors the production of the transpeptidation reaction product to probe the substrate specificity of these isoforms, as well as to screen potential small molecule inhibitors (Kruger *et. al.*, in preparation). In this assay we chose fluorescein and rhodamine dyes as the FRET donor/acceptor pair because of their high efficiency of energy transfer, their extended Forster radius, and because activated forms of the 5(6)-carboxylic acid analogues, although expensive, were available commercially for attachment to amino acid side chains (Fig. 2). This assay relies on the gain in fluorescence due to intramolecular FRET in the transpeptidation product resulting from the covalent attachment of two individually labeled peptide substrates (Scheme 1). However, inefficiencies associated with off-resin labeling of the peptides with activated rhodamine and fluorescein succinimido esters or isothiocyanates coupled with the expense of purified carboxyrhodamine ($> \$1000\ g^{-1}$) severely limited the scale to which peptides could be efficiently produced to a few milligrams. Since the *S. aureus* Sortase exhibits a relatively high K_M for its LPXTG-containing peptide substrate, performing a full detailed kinetic analysis was prohibited by material requirements and reagent expenses. Therefore we devised a general solid-phase synthesis of these substrates in which carboxylic acid derivatives of the FRET probes were site specifically attached to the sidechain of orthogonally-protected Lys residues during the peptide assembly process (Scheme 2). In addition, we discovered an inexpensive route to 5- and 6-carboxyrhodamine that facilitated the economic production of gram scale quantities of peptides labeled with these fluorescent probes.

There are several recent examples in the literature of on-resin assembly of fluorescently labeled peptides to internal Lys residues using Alloc or Mtt orthogonal deprotection strategies.[10–15] Building upon these precedents, we chose to employ the Dde protecting group for the Lys N^{ε} side chain amine to both avoid known difficulties associated with on-resin Pd(0)-catalyzed removal of Alloc groups and to avoid unnecessary exposure of the peptide resin to TFA as would be encountered during Mtt group removal. The Lys(Dde) group is stable during Fmoc solid-phase synthesis and is efficiently removed by a solution of 2% hydrazine in DMF. We also chose to introduce

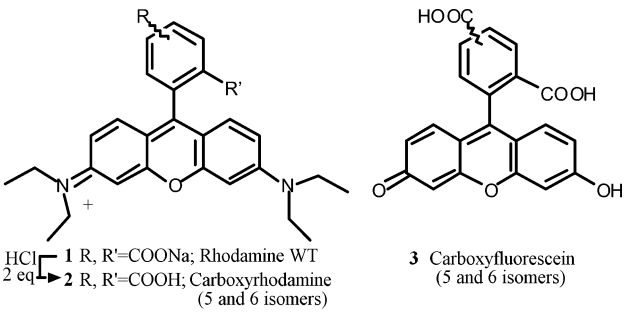


Fig. 2 Structures of carboxylic acid derivatives of rhodamine and fluorescein FRET probes used in this study and the conversion of rhodamine WT to its free acid.

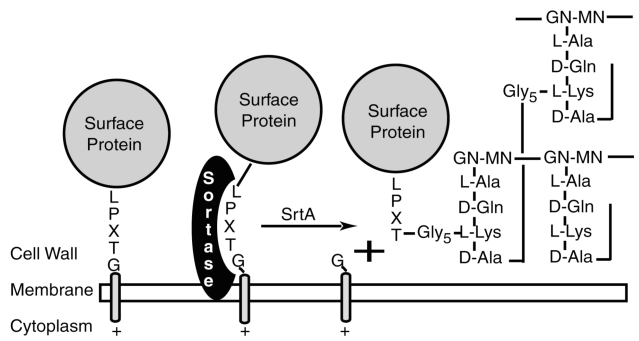
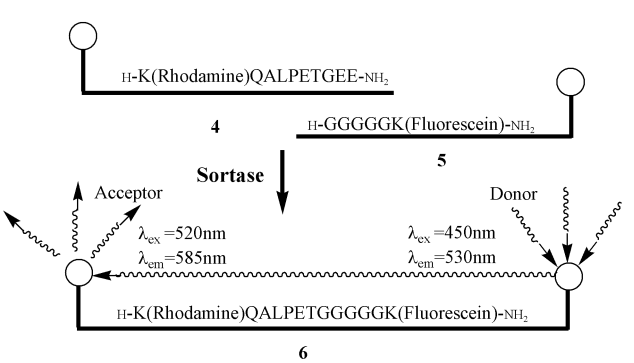


Fig. 1 The *S. aureus* Sortase transpeptidase reaction.



Scheme 1 Application of intramolecular FRET to analysis of the Sortase-catalyzed transpeptidase reaction.

† Electronic supplementary information (ESI) available: HPLC and analytical data. See http://www.rsc.org/suppdata/cc/b2/b206303d/

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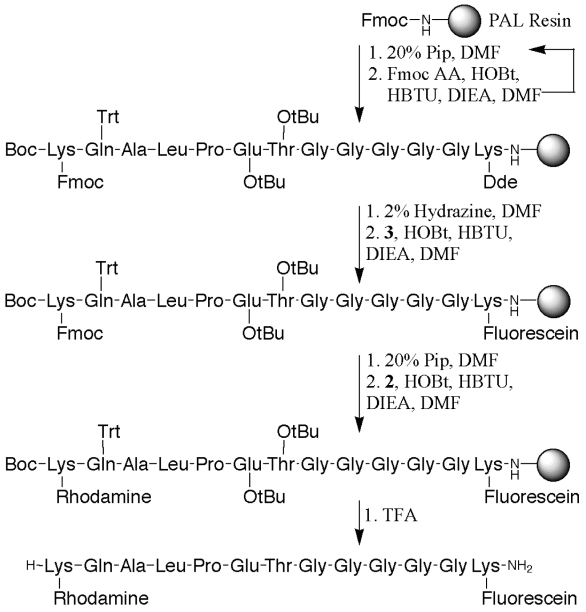
the rhodamine and fluorescein fluorophores into the peptide sequence using their 5-carboxy derivatives with standard uronium-based amide bond forming reagents (*e.g.* HBTU). Thus, these FRET peptides could be assembled entirely by automated means if desired. Automation would also facilitate future synthesis of parallel and combinatorial libraries for substrate specificity studies of SrtA. However, since a four-fold excess of amino acid and coupling reagents are typically required for 0.1–0.25 mmol scale automated syntheses, an economical source of 5-carboxyrhodamine needed to be identified.

Although the free acid of 5(6)-carboxyrhodamine (mixture) is expensive, its sodium salt is extremely inexpensive (*ca.* < \$1 g^{-1}). Marketed as Rhodamine WT (for water tracer) (**1**), it is commercially available as a 20% aqueous solution (cost \$12.60 kg^{-1}, Abbey Color, Philadelphia, PA). Acidification of **1** with two equivalents of HCl precipitated a mixture of the 5- and 6-carboxy isomers (**2**) that were preparatively separated by reversed-phase HPLC[16,17] and characterized by HR-MS and NMR (Fig. 2 and ESI†).

With this in hand, we prepared 5-carboxyrhodamine-containing peptide **4** by automated solid-phase methods on a 0.25 mmol scale. In order to introduce the rhodamine label away from the Sortase LPXTG recognition sequence, Boc-Lys(Fmoc)-OH was added as the last residue. Following piperidine deprotection, 5-carboxyrhodamine (a 4-fold molar excess) was coupled to the Lys ε-NH_2 using HBTU/HOBt/DIEA in DMF. The peptide was cleaved with TFA–H_2O (95:5 v/v), and the crude peptide was purified to homogeneity by HPLC and characterized by MS (ESI†). For fluorescein-containing peptide **5**, the fluorophore was introduced internally into the sequence *via* an Fmoc-Lys(Dde)-OH residue. Following linear assembly of the peptide and removal of the Dde protecting group with 2% hydrazine in DMF, the resulting amine was acylated with 5-carboxyfluorescein using HBTU/HOBt/DIEA in DMF, then cleaved, purified by HPLC and characterized by MS (ESI†). The fluorescence excitation and emission spectra of **4** and **5** were similar to the parent fluorophores (Fig. 3). In addition, Scheme 2 shows the assembly of the doubly labeled transpeptidation product **6** (ESI†), which was prepared in the same manner as peptides **4** and **5**.

As predicted, the SrtA transpeptidation product **6** exhibited FRET as evidenced by the strong quenching of the fluorescein donor's emission at 530 nm and the increase in the emission of the rhodamine acceptor at 585 nm (Fig. 3). Since, by 2D-NMR analysis, peptide **6** exhibited modest β-hairpin structure that



Scheme 2 Representative orthogonal solid-phase synthesis of peptides containing pendant FRET probes.

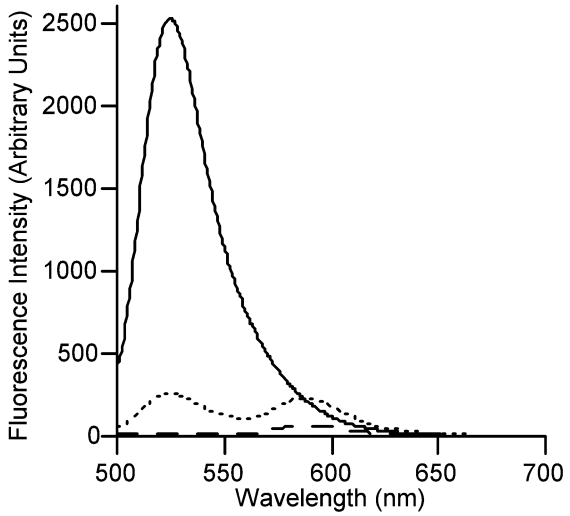


Fig. 3 Fluorescence emission spectra of peptide substrate **4** (dashed), peptide substrate **5** (solid), and the doubly labeled transpeptidation product **6** (dotted) after fluorescein excitation at 450 nm. *Conditions*: 1.0 μM peptide, 10 mM phosphate (pH 7.5), 6 M Gnd·HCl.

placed both donor and acceptor probes near each other, deleterious intramoleular FRET self-quenching observed in water was eliminated by obtaining fluorescence emission spectra in 10 mM phosphate buffer (pH 7.5) containing 6 M guanidine hydrochloride.

This general method of synthesizing peptide substrates containing rhodamine and fluorescein FRET donor/acceptor pairs placed at site-specific locations within a peptide sequence is advantageous over existing methods because it is facile, amenable to preparative scales, and is economical since rhodamine WT is an inexpensive source for 5-carboxyrhodamine. This method can also be easily extended to synthesis of protease substrates for FRET analysis and to the creation of parallel or combinatorial substrate libraries for protease or transpeptidase substrate specificity studies.[14]

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